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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53 (c).

INVENTOR(S)		
Given Name (first and middle (if any))	Family Name or Surname	Residence (City and either State or Foreign Country)
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☐ Additional inventors are being named on the ___ separately numbered sheets attached hereto

TITLE OF THE INVENTION (280 characters max)

A Marker Specific for Escherichia coli Serotypes 0157:H7; O157:NM and O55:H7

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ENCLOSED APPLICATION PARTS (check all that apply)

☑ Specification Number of Pages 17 ☑ Small Entity Statement

☑ Drawing(s) Number of Sheets 3 ☐ Other (specify)

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT (check one)

☑ A check or money order is enclosed to cover the filing fees CHQ#528

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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

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☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

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Date 14/06/1999

REGISTRATION NO. (if appropriate) 40,261

Docket Number: 6580-164

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C., 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C., 20231.

B&P File No. 6580-164 /MG

BERESKIN & PARR **UNITED STATES**

Title: A marker specific for *Escherichia coli* serotypes O157:H7; O157:NM and O55:H7

Inventors: Shu Chen and Renlin Xu

B&P File No. 6580- 164/MG

Title: A marker specific for *Escherichia coli* serotypes O157:H7; O157:NM and O55:H7

FIELD OF THE INVENTION

This invention relates to a novel DNA marker specific for *E. coli* serotypes O157:H7, O157:NM and O55:H7 and the use of the marker in developing assays to detect these serotypes of E. coli in a sample.

BACKGROUND OF THE INVENTION

E. coli O157:H7 is a food-borne human pathogen causing a spectrum of diseases including diarrhea, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). Development of nucleic-based assays for rapid detection of *E. coli* O157:H7 has been challenging due to a lack of specific DNA markers for this organism. The reported DNA markers for *E. coli* O157:H7 have one or more of the following limitations or drawbacks: being non-specific, so that multiplex PCR assays need to be used; and containing only one base pair mutation, limiting its use in assay development. Consequently, there is a need in the art to provide novel markers for E. coli O157:H7 and related serotypes.

SUMMARY OF THE INVENTION

The present inventors have prepared a novel marker specific for E. coli serotypes O157:H7, O157:NM and O55:H7. The DNA sequence of the marker has a total of 1583 nucleotides and has no significant homology to any known DNA sequences. Accordingly, in one aspect, the present invention provides an isolated nucleic acid sequence having the sequence shown in Figure 1 or a diagnostic fragment thereof.

The marker of the invention can be used to develop probes or primers that can be used to detect E. coli serotypes O157:H7; O157:NM and O55:H7 in a sample. In particular, the marker has been demonstrated to be useful in designing primers for PCR assays for specific detection of the *E. coli* serotypes. As the specific DNA sequences of this invention are not homologous to previously known sequences, various specific PCR assays can be developed with only one primer pair. In addition, other nucleic

acid-based assays such as DNA chip or biosensor assays can also be developed without the restriction in using a very limited region of a marker. The invention can be used by biotechnology companies and by central and diagnostic laboratories who are interested in developing nucleic acid based assays.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in relation to the drawings in which:

Figure 1 shows the nucleotide sequence of the 1583 bp marker of the invention.

Figure 2 shows the nucleotide sequence of a 360 bp fragment of the marker.

Figure 3 is a gel electrophoresis of PCR products amplified using the PCR assay from DNA preparations of representative *E. coli* serotypes: lanes 1, 2, O157:H7; lanes 3, 4, O157:NM; lanes 5 - 9, O145:NM; lanes 10, 11, O55:H7; lane 12, O26:H11; lane M, 100 bp DNA ladder.

DETAILED DESCRIPTION OF THE INVENTION

I. **MARKER**

As hereinbefore mentioned, the present inventors have prepared a novel DNA marker specific for E. coli serotypes O157:H7, O157:NM and O55:H7.

The marker was identified and isolated using a relatively new marker technology, fluorescent amplified fragment length polymorphism (FAFLP) and DNA sequencing. The DNA sequence of the marker has a

total of 1583 nucleotides and has no significant homology to any known DNA sequences.

Accordingly, in one aspect, the present invention provides an isolated nucleic acid molecule having the sequence shown in Figure 1 or a diagnostic fragment thereof.

The term "isolated" refers to a nucleic acid molecule substantially free of cellular material or culture medium when produced by recombinant DNA techniques or chemical precursors when chemically synthesized.

The term "diagnostic fragment" means any fragment of the marker shown in Figure 1 that is useful in a diagnostic assay to detect E. coli serotypes O157:H7; O157:NM and O55:H7. The diagnostic fragment includes fragments that can be used as primers in PCR assays and fragments that can be used as probes in detection assays. The diagnostic fragments of the invention will not cross react with bacteria other than E. coli serotypes O157:H7; O157:NM and O55:H7.

The invention also includes (1) sequences that are complimentary to the sequence shown in Figure 1 or a diagnostic fragment thereof and (2) sequences that hybridize to the sequence shown in Figure 1 or a diagnostic fragment thereof. Such sequences are also useful in developing diagnostic assays to detect E. coli serotypes O157:H7; O157:NM and O55:H7.

II. USES OF THE MARKER

As previously mentioned, the isolation of the novel marker for E. coli serotypes O157:H7; O157:NM and O55:H7 allows the development of diagnostic assays that can be used to detect the serotypes in a sample. The sample can be any sample, including but not limited to, clinical, food and environmental samples.

(a) Primers

The present invention includes the preparation of nucleic acid primers based on the sequence of the marker shown in Figure 1. Accordingly, the present invention provides an isolated nucleic acid

primer having a sequence that is complimentary to a portion of a nucleic acid sequence shown in Figure 1.

In one embodiment, the primer has the sequence shown in Table 3.

Using the primers illustrated in Table 3 in a PCR assay, the inventors have demonstrated that these primers are specific for the E. coli serotypes O157:H7; O157:NM and O55:H7 but are not specific for 119 other E. coli strains belonging to 60 serotypes and 59 isolates belonging to 44 non-E. coli species (see Example 1).

Accordingly, the present invention provides a method of detecting the presence or absence of E. coli serotypes O157:H7; O157:NM and O55:H7 in a sample comprising (a) isolating DNA from the sample; (b) amplifying the isolated DNA with a primer of the invention and assaying for amplified sequences, wherein the presence of an amplified sequence indicates that the sample contains one of E. coli serotypes O157:H7; O157:NM and O55:H7.

The DNA sequences are preferably amplified in step (b) using a Polymerase Chain Reaction (PCR).

The conditions which may be employed in the methods of the invention using PCR are those which permit hybridization and amplification reactions to proceed in the presence of DNA in a sample and appropriate complementary hybridization primers. Conditions suitable for the polymerase chain reaction are generally known in the art. For example, see M.A. Innis and D.H. Gelfand, PCR Protocols, A guide to Methods and Applications M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White eds, pp3-12, Academic Press 1989, which is incorporated herein by reference. Preferably, the PCR utilizes polymerase obtained from the thermophilic bacterium *Thermus aquatics* (Taq polymerase, GeneAmp Kit, Perkin Elmer Cetus) or other thermostable polymerase may be used to amplify DNA template strands.

The primers may be labelled with detectable markers which allow for detection of the amplified products. Suitable detectable markers

are radioactive markers such as P-32, S-35, I-125, and H-3, luminescent markers such as chemiluminescent markers, preferably luminol, and fluorescent markers, preferably dansyl chloride, fluorcein-5-isothiocyanate, and 4-fluor-7-nitrobenz-2-axa-1,3 diazole, enzyme markers such as horseradish peroxidase, alkaline phosphatase, ß-galactosidase, acetylcholinesterase, or biotin.

It will be appreciated that techniques other than PCR such as the Ligase Chain Reaction (LCR) and NASBA may be used to amplify a nucleic acid molecule of the invention (Barney in "PCR Methods and Applications", August 1991, Vol.1(1), page 5, and European Published Application No. 0320308, published June 14, 1989, and U.S. Serial No. 5,130,238 to Malek).

(b) Probes

The present invention also includes the preparation of nucleic acid probes based on the sequence of the marker shown in Figure 1. Accordingly, the present invention provides an isolated nucleotide probe having a portion of a sequence shown in Figure 1.

The probes can be used to detect the presence or absence of E. coli serotypes O157:H7; O157:NM and O55:H7 in a sample.

Accordingly, the present invention also relates to a method of detecting the presence of a nucleic acid molecule associated with E. coli serotypes O157:H7; O157:NM and O55:H7 in a sample comprising (a) contacting the sample under hybridization conditions with one or more of nucleotide probes which hybridize to the nucleic acid molecules and are labelled with a detectable marker, and (b) determining if there is hybridization between the nucleic acid molecules in the sample and the nucleotide probes wherein the presence of hybridization indicates that the sample contains one of E. coli serotypes O157:H7; O157:NM and O55:H7.

Hybridization conditions which may be used in the methods of the invention are known in the art and are described for example in Sambrook J, Fritch EF, Maniatis T. In: Molecular Cloning, A Laboratory Manual,1989. (Nolan C, Ed.), Cold Spring Harbor Laboratory Press, Cold

Spring Harbor, NY. The hybridization product may be assayed using techniques known in the art. The nucleotide probe may be labelled with a detectable marker and the hybridization product may be assayed by detecting the detectable marker or a detectable change produced by the detectable marker.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

Example 1

AFLP marker identification

The marker was initially identified as a 360 bp fluorescent fragment (Figure 2) using the FAFLP method (Vos et al., 1995. Neucleic Acids Research, Vol. 23, pp. 4407-4414). The FAFLP analysis was performed by the selective amplification of restriction fragments from a digest of total genomic DNA with restriction enzymes *EcoR* I and *Mse* I. The DNA sequences of the selective primer pair that produced the polymorphic marker are as shown in Table 1. Among 163 *E. coli* strains tested that belonged to 40 serotypes (Table 2), only serotypes O157:H7, O157:NM, O145:NM and O55:H7 contained the 360 bp fragment.

DNA sequencing

The target fragment was directly isolated from the AFLP gel using ABI 377 automated DNA Sequencer and re-amplified by polymerase chain reaction (PCR), and then sequenced. The DNA sequence of the 360 bp fragment is shown in Figure 2. A 1223 bp downstream region continued from the AFLP fragment was further sequenced using a single primer walking method with strains of *E. coli* serotypes O157:H7, O157:NM, O55:H7 and O145:NM. Four mutations (C-T, A-G, T-A and G-A) in the region were identified in the strain of serotype O145:NM. The complete sequence of the 1583 bp fragment is shown in Figure 1. The result of BLAST search showed that the DNA sequences of the 1583 bp fragment had no significant homology to 400 sequences of the *E. coli* genome and 400,635 known sequences in the GenBank, EMBL, DDBJ, PDB

DNA databases. The DNA sequences of the 1583 bp fragment were also analyzed by a software program, GeneWorks 2.5; a potential open reading frame (ORF) was identified within the sequences of 1043 - 90 nt.

Marker application

The DNA fragment has been useful as a marker in developing a PCR assay for specific detection of *E. coli* serotypes O157:H7, O157:NM and O55:H7. The DNA sequences of the primers used in the PCR assay are provided in Table 3. Primer O157-F contains one mismatch to the sequences of O157:H7/O157:NM for eliminating possible cross reaction from the sequences of O145:NM. The primer pair results in a 560 bp PCR product under the following conditions. An amplification reaction mixture (20 μL) contained 10mM Tris-HCl and 50 mM Kcl (pH 8.3), 1.2 mM $MgCl_2$, 200 μM each of dATP, dCTP, dGTP and dTTP, 0.4 pmol/μL each of the primers, 0.5 unit of *Taq* DNA polymerase (Perkin-Elmer) and 5 ul of DNA template. The thermal cycling conditions (GeneAmp 9600 PCR System, Perkin-Elmer) were as follows: initial denaturation at 94°C for 3 min; 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and denaturation at 72°C for 45 sec; incubation at 72°C for 5 min. PCR products were visualized on a 1.6% agarose gel after staining with ethidium bromide.

The specificity of the primers and the PCR assay has been evaluated with a total of 119 *E. coli* strains belonging to 60 serotypes (Table 4) and 59 isolates belonging to 44 non-*E. coli* species (Table 5). The 560 bp specific PCR product appeared only with strains of serotypes O157:H7, O157:NM and O55:H7 (Figure 3) but not the strains of the other serotypes of E. coli or other bacterial strains tested. The PCR assay can be used for the detection of *E.coli* O157:H7 in food, clinical and environmental samples.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various

modifications and equivalent arrangements included within the spirit and scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table 1

DNA sequences of the selective primers that produced the AFLP marker

Primer	Oligo-nucleotide sequence
EcoR I-C	5'-GACTGCGTACCAATTCC-3'
Mse I-G	5'-GATGAGTCCTGAGTAAG-3'

Table 2

E. coli strains screened for the presence of the O157:H7 marker in AFLP analysis

Serotype	No. of strains	Serotype	No. of strains	Serotype	No. of strains
O?:H7	1	O91:H21	5	O139:K82	1
O?:H8	1	O91:NM	2	O145:NM	2
O5:NM	4	O98:NM	1	O153:H25	3
O7:H4	4	O103:H2	24	O153:H31	1
O8:H19	1	O111:H8	1	O156:H7	1
O22:H8	5	O111:NM	5	O156:NM	1
O26:H11	6	O113:H4	1	O157:H7	56
O39:H49	1	O113:H21	2	O157:H19	1
O46:H38	1	O115:H8	1	O157:H25	1
O55:H7	3	O118:H16	1	O157:NM	2
O76:H25	1	O121:H7	1	O163:H19	1
O80:NM	3	O127:H6	1	O163:NM	1
O88:H25	1	O128:B12	1		
O91:H14	3	O132:NM	11		

Table 3

DNA sequences of the primers specific for

E. coli O157:H7, O157:NM and O55:H7

Primer	Oligo-nucleotide sequence	Location within the marker sequence
O157-F (22-mer)	5'-CGGTTTAATGGCTTGTTGTGCT-3'	596 - 618
O157-R (19-mer)	5'-ATGCCATTAAACCGGTGGC-3'	1136 - 1155

Table 4

E. coli strains tested for the presence of the O157:H7 marker in the PCR assay

Serotype	No. of strains	Serotype	No. of strains	Serotype	No. of strains
O?:H2	1	O55:H7	6	O126:H8	1
O?:H7	1	O76:H25	1	O127:H6	1
O?:H8	1	O80:NM	2	O128:B12	1
O?:H19	1	O84:H2	1	O132:NM	2
O?:H21	1	O88:H25	1	O136:H16	1
O2:H29	1	O91:H14	3	O139:K82	1
O5:NM	2	O91:H21	3	O142:H38	1
O7:H4	2	O91:NM	2	O145:NM	5
O8:H?	1	O98:NM	1	O153:H25	3
O8:H9	1	O103:H2	5	O153:H31	1
O8:H19	3	O111:H8	2	O153:NM	1
O15:H27	1	O111:NM	5	O156:H7	1
O15:NM	1	O113:H4	1	O156:NM	1
O22:H8	2	O113:H21	2	O157:H7	15
O26:H11	5	O115:H8	1	O157:H19	1
O26:NM	1	O116:H21	1	O157:H25	2
O38:H21	1	O118:H16	1	O157:NM	6
O39:H49	1	O121:H6	1	O163:H19	1
O45:H2	1	O121:H7	2	O163:NM	2
O46:H38	1	O121:H19	1	O165:NM	1

Table 5

Non-E. coli species tested for the presence of the O157:H7 marker in the PCR assay

Species	No. of isolates
Acinetobacter calcoaceticus	1
Aeromonus hydrophila	2
Bacillus cereus	2
Bacillus circuluris	1
Bacillus parabrevis	1
Bacillus subtilis	1
Citrobacter diversus	1
citrobacter freudii	1
Campylobacter	2
Campylobacter coli	2
Campylobacter jejuni	1
Canadida albicus	1
Enterobacter cloacae	1
Enterobacter amnigenus	1
Enterococcus fecalis	2
Edwardsiella tarda	1
Kluyvera ascovbata	1
Listeria monocytogenes	1
Listeria ivarovii	1
Lactobacillus brevis	1
Lactobacillus planterum	1
Moraxella osloensis	1
Proteus vulgaris	1
Proteus mirabilis	1
Pseudomonus aeruginosa	1
Salomonella tryphimurium	4
Salmonella heidelberg	1
Salmonella thompson	1
Salmonella newport	1
Salmolella hadar	2
Salmonella infantis	2
Salmonella schwarzengrund	2
Salmonella choleraesuis var.kunzendorf	2
Shigella flexneri	1
Shigella sonnei	1
Straphylococcus aureus	4
Straphylococcus epidermidis	1
Straphylococcus haemolyticus	1
Straphylococcus lugdenersis	1
Straphylococcus intermedium	1
Streptococcus pyogenes	1
Streptococcus bovis	1
Xanthomonas malitophilia	1
Yersinia enterocolitica	1

We Claim:

1. An isolated nucleic acid molecule having the sequence shown in Figure 1 or a diagnostic fragment thereof.

2. An isolated nucleic acid molecule having (1) a sequence that is complimentary to the sequence claimed in claim 1 or (2) a sequence that hybridizes to a sequence claimed in claim 1.

3. An isolated nucleic acid primer having a sequence that is complimentary to a portion of a nucleic acid sequence claimed in claim 1.

4. A nucleic acid primer according to claim 3 wherein the primer has the sequence (a) 5'-CGGTTTAATGGCTTGTTGTGCT-3' or (b) 5'-ATGCCATTAAACCGGTGGC-3'.

5. An isolated nucleotide probe having a portion of a sequence claimed in claim 1.

6. A method of detecting the presence or absence of E. coli serotypes O157:H7; O157:NM and O55:H7 in a sample comprising (a) isolating DNA from the sample; (b) amplifying the isolated DNA with a primer as claimed in claim 3 and (c) assaying for amplified sequences, wherein the presence of an amplified sequence indicates that the sample contains one of E. coli serotypes O157:H7; O157:NM and O55:H7.

7. A method according to claim 6 wherein the primer is according to claim 4.

8. A method according to claim 6 wherein the DNA sequences are amplified in step (b) using a Polymerase Chain Reaction.

9. A method of detecting the presence of a nucleic acid molecule associated with E. coli serotypes O157:H7; O157:NM and O55:H7 in a sample comprising (a) contacting the sample under hybridization conditions with one or more of nucleotide probes which hybridize to the nucleic acid molecules and are labelled with a detectable marker, and (b) determining if there is hybridization between the nucleic acid molecules in the sample and the nucleotide probes, wherein the presence of hybridization indicates that the sample contains one of E. coli serotypes O157:H7; O157:NM and O55:H7.

10. A method according to claim 9 wherein the nucleotide probe is according to claim 5.

BP File No. 6580-164/MG

ABSTRACT OF THE DISCLOSURE

A novel DNA marker specific for E. coli serotypes O157:H7; O157:NM and O55:H7 is disclosed. The isolation of the marker allows the development of diagnostic assays that can be used to detect the serotypes in the sample. In particular, the marker can be used to prepare nucleic acid primers and nucleotide probes based on the sequence of the marker.

Applicant or Patentee: _____ Attorney's
Serial or Patent No.: N/A Docket No:
Filed or Issued: N/A
For: A Marker Specific for *Escherichia coli* serotypes 0157:H7; 0157:NM and

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(b)) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled A Marker Specific for *Escherichia coli* serotypes 0157:H7; 0157:NM and 055: H7 described in

[X] the specification filed herewith
[ ] application serial no. _____, filed _____.
[ ] patent no. _____, issued _____.

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF INVENTOR	NAME OF INVENTOR	NAME OF INVENTOR
Shu Chen	Renlin Xu	
(PRINT NAME HERE)	(PRINT NAME HERE)	(PRINT NAME HERE)
Signature of Inventor	Signature of Inventor	Signature of Inventor
Date June 14, 1999	Date June 14, 1999	Date

Applicant or Patentee: Shu Chen, Renlin Xu
Serial or Patent No.: N/A
Filed or Issued: N/A
Attorney Docket No. _____
For: A Marker Specific Mark... *Escherichia coli* Serotypes 0157:H7; 0157:NM and ...:H7

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27 (d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

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I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) or (b) of Title 35, United States Code with regard to the invention entitled **GENETIC MARKERS FOR *ESCHERICHIA COLI* 0157:H7** by **SHU CHEN, RENLIN XU** described in

[X] the specification filed herewith
[ ] application serial no. _____, filed _____.
[ ] patent no. _____, issued _____.

I hereby declare the rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a non-profit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME _____
ADDRESS _____ [ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

NAME _____ ADDRESS _____
[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28 (b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Connie Hearty
TITLE OF PERSON OTHER THAN OWNER Director of Business Development
ADDRESS OF PERSON SIGNING University of Guelph, Guelph, Ontario, N1G 2W1
SIGNATURE _____ DATE June 11/99

FIGURE 1

DNA sequence of the 1583 bp fragment

```
                CTGCACCTTT TTTTGCTGTG CCTTTGGGAT CCGCGCGAAT GCGCCCAGAA      50
                AACCATCAGA AAGTGCCACC TTGGGCGTTT GCACCGTCAT TTGTCACCTC     100
    5           CGGACAGATG TTGCAATGTA TTTTCGTCAA TTGGCCCGAA TGCAACGTGC     150
                CAACCCCTGG TTGTGAATAA TGGAATAAAT TCTTGATTAT CAATAATCAA     200
                TGCCCATTTT TGTGATGGCC AGGCGAGATC GGCTTCCGCA ATAATTTCAC     250
                CTTCATCATT TTGCAATTCG TAGCCGAGAG TCGGCGCAGA GAGAGAATGA     300
                GACTGAAGCA ACGCGATCTC TTCAGCGCTT AATAAGCCAA ACTCAATAAT     350
    10          CTCAGCCCAA CTCTCATCAG TCTCTGGTAC TGCCTCTGGC GTTGCCGAAA     400
                CTTTAGTGTC AACGCTTTCC TGCGGTAAAT GCACGGCTTT ACGGCTGGTA     450
                AAGGTCATAT CCGGCAGGAA CTGTAACAGG TTTACCAGTC GCCAGAAGCC     500
                ATTGAGTCCC GCTTCATAAC CGTCATCCTG CGTATAGCGA TCGTCAAAAC     550
                AGATATGCAA ACGCAGCCAG CTGCGCATTT GTGCGACGCT GGTCGTCGGT     600
    15          TTAATGGCTT GTTGTGGTAA CACCGAAGCC AGCTCAATAA ATTGCTGCGA     650
                TGAGTTACAG CTATCGAGTA AACCACCAAA GACAAACGGT TCGTCTGGCA     700
                AAAGCTCTGC TAGCCGGCAA GGTGGCGCGT TTTCCTGCAT TTCATAGGGA     750
                TACTTTTGTT TCGTTCCCGC GTCCTGTGAG TTCTTCACAT CCAACCACAC     800
                CCAGGCATGT GCAGCAGCCA TTCTCTGCCA TAACTGCGTT CTTCCGCCCG     850
    20          GATCGGCGAG ATAATCCAGG AGCAGTGCAA AACTGTTGCG TTCCCGGAAG     900
                GACGCTTCAA GGGCCGCAAA GTTGGTATCA TGAAACAGGT TATAAAACTT     950
                AGGCTGTTTC ATATCCGGGT TATGCCCAAG TCCCAGGACA TCCTGAACAT    1000
                GTTTAAGCCC TGGCTCTTTG AGATCGGCCA CGTGAGTGTC CACACCCAGA    1050
                AATTACGGCT ATCCCGAATC GCCTGGCGTT TTTGCACATC CTCTGCGACG    1100
    25          CTATTTTTGT GGAACGCAAA GCCATCAAGG AAAATAGCCA CCGGTTTAAT    1150
                GGCATCAGAC TGCATAAGCG GATATAGCAC GTAATCCGGG CGACAAGGGA    1200
                TCCCCACCGC CTCTTTAGCG CCTAAATCCA CCTGAGCTTT AAGATGCCAG    1250
                CTCATCATCG GTTCGCTGCG GGTGTTAATA ATCCACCCAG CGCCCTGATG    1300
                CGCGTAACTG CGACTGACAA TCAGGTTTTT ATTTTCTTGC AGGCAGCTAA    1350
    30          TAAACCGTTT TTCCAGCTCA CTGCCCATCA TGGCTTCGAG GGAAATGTTT    1400
                TTGATGGAAT CAATTACCCG TATCCAATCT CTTGCCTGCA AAATTTTCGC    1450
                CAGCAAGAGA CGCGCCTGAT CGCGGGAGAC ATACTTCATG CGCCCACGGT    1500
                CACGGTAAGC GTAAACGCAA CGATAACAAC CATCTTTATG CGTATCATTG    1550
                TTGCAACTGC ATTCCACTAT TGCCTGATAG GCC                      1583
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FIGURE 2

DNA sequence of the 360 bp AFLP marker

	CTGCACCTTT TTTTGCTGTG CCTTTGGGAT CCGCGCGAAT GCGCCCAGAA	50
5	AACCATCAGA AAGTGCCACC TTGGGCGTTT GCACCGTCAT TTGTCACCTC	100
	CGGACAGATG TTGCAATGTA TTTTCGTCAA TTGGCCCGAA TGCAACGTGC	150
	CAACCCCTGG TTGTGAATAA TGGAATAAAT TCTTGATTAT CAATAATCAA	200
	TGCCCATTTT TGTGATGGCC AGGCGAGATC GGCTTCCGCA ATAATTTCAC	250
	CTTCATCATT TTGCAATTCG TAGCCGAGAG TCGGCGCAGA GAGAGAATGA	300
10	GACTGAAGCA ACGCGATCTC TTCAGCGCTT AATAAGCCAA ACTCAATAAT	350
	CTCAGCCCAA	360

FIGURE 3

M 1 2 3 4 5 6 7 8 9 10 11 12